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A Thesis Submitted to the
Yale University School of Medicine
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Degree of Doctor of Medicine

By
Judd Warren Landsberg
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Effect of Manipulating Intraamygdala Levels of cGMP on Fear Conditioning.

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Long-term potentiation (LTP) describes the persistent, activity dependent increase in synaptic efficacy which has been seen in many vertebrate brain areas both in vitro and in vivo and is considered by many to be a promising model of learning and memory. Recent in vitro studies of the CA1 region of the rat hippocampus have implicated cyclic guanosine 3',5'-monophosphate (cGMP) as an important second messenger in the formation of LTP. LY-83583, an inhibitor of cGMP production, was shown to block LTP, while addition of 8 Br-cGMP, the membrane permeable cGMP analogue, produced LTP. We conducted the following experiments to investigate the effect of intraamygdala infusion of LY-83583 and 8 Br- cGMP on fear conditioning, an example of in vivo learning, as measured behaviorally by fear potentiated startle in male Sprague - Dawley rats. Bilateral intraamygdala infusion of 2.5 nmol LY-83583, 6 min prior to 30 light - shock (0.6mA) pairings, failed to block the acquisition of conditioned fear. Bilateral intraamygdala infusion of 50nM 8 Br-cGMP, 6 min prior to 30 light -shock pairings, failed to enhance the acquisition of either weak fear conditioning or strong fear conditioning. Overall, we were not able to manipulate the acquisition of fear potentiated startle by intraamygdala infusion of these two agents recently shown to impair and enhance LTP in vitro.

Introduction

Long-term potentiation (LTP) describes the persistent, activity dependent increase in synaptic efficacy which has been seen in many vertebrate brain areas both in vitro and in vivo (Nicoll et al 1988). Because LTP is considered by many to be a promising model of learning and memory, understanding the molecular events involved in LTP and the roles played by the presynaptic and postsynaptic neurons is an area of active investigation. Currently it is felt that LTP is a two stage phenomenon having both an induction and a maintenance phase.

Induction is believed to be largely a postsynaptic cell phenomenon. It is felt that glutamate released by presynaptic neurons binds to two distinct targets on postsynaptic neurons, NMDA and non - NMDA receptors. The non - NMDA receptors, upon binding glutamate, allow a small inward Na+ current, which produces an excitatory postsynaptic potential (EPSP). When glutamate binds to the postsynaptic NMDA receptors one of two events occurs. If the postsynaptic cell is at resting membrane potential, the NMDA receptor's ion channel will be blocked by a Mg++ ion and nothing occurs. However, if the postsynaptic cell has been recently depolarized, the Mg++ block will be removed and binding of glutamate leads to a Ca++ influx which triggers LTP (Fig. 1). It has been shown in vitro that NMDA receptor antagonists, Ca++ chelating agents, and artificial hyperpolarization of postsynaptic neurons all block LTP. Similarly intracellular micro-injection of Ca++ into postsynaptic neurons has been shown to trigger LTP (Nowak et al 1992). In short, the second messenger in the induction of LTP is believed to be the postsynaptic, NMDA receptor mediated, Ca++ influx.

Maintenance of LTP was shown by Bekkers and Stevens (Bekkers et al 1990) to be largely a phenomenon of the presynaptic neuron accomplished by an increase in release of neurotransmitter. Implicating the presynaptic cell in the maintenance of LTP implied the existence of a retrograde signal linking the postsynaptic cell to the presynaptic cell, and LTP induction to LTP maintenance. Searching for a possible retrograde messenger, Madison and Schuman (1991) demonstrated that NO synthase inhibitors blocked LTP when injectcd directly into postsynaptic neurons. Recently, Kandel's group in a series of experiments have implicated guanylate cyclase and cyclic guanosine 3',5'-monophosphate (cGMP) production as the target of the retrograde signal. Their in vitro studies of the CA1 region of the rat hippocampus have demonstrated that LY-83583, an inhibitor cGMP production, blocked LTP (Zhuo et al 1994). Furthermore, addition of the membrane permeable cGMP analogue, 8 Br-cGMP, paired with weak stimulation of presynaptic neurons produced LTP (Zhuo et al 1994). As predicted, inhibition of LTP by both NO synthesis inhibitors and LY-83583 was over come by administration of 8 Br- cGMP(Zhuo et al 1994). These data implicated NO as the retrograde messenger, and cGMP as the presynaptic second messenger of LTP maintenance (Fig. 1).

We have previously demonstrated that NMDA antagonists that block the induction of LTP in vitro will similarly block learning, specifically conditioned fear, when infused directly into the amygdala (Miserendino et al 1990). We conducted the following experiments to investigate the effect of manipulating intraamygdala levels of cGMP on the acquisition of conditioned fear in male Sprague - Dawley rats as measured behaviorally by fear potentiated startle. Fear potentiated startle is a paradigm whereby the acoustic startle

reflex is increased when elicited in the presence of a neutral stimulus (e.g. light) previously paired with an aversive stimulus such as shock (Davis & Astrachan, 1978), as illustrated in Fig. 6. Under normal circumstances the magnitude of the increase in the startle reflex over baseline is related to the strength of the fear conditioning.

The first experiment examined the effect of intraamygdala infusion of LY-83583, an inhibitor of cGMP production, (Brandt et al 1991) on the acquisition of conditioned fear as measured by fear potentiated startle. To demonstrate correct cannula placement and effective drug delivery animals that previously received LY-83583 were given an intraamygdala infusion of NBQX, a glutamate receptor antagonist known to block the expression of fear potentiated startle when infused directly into the amygdala (Kim et al 1993), and re-tested for fear potentiated startle.

Next we established an intensity response curve for various foot shock intensities on the acquisition of conditioned fear to determine a training intensity where only weak fear conditioning occurs. Then we examined the effect of intraamygdala infusion of 8 Br-cGMP on weak (0.2mA shock) fear conditioning. Finally we investigated the effects of an intraamygdala infusion of 8 Br-cGMP on strong (0.6mA shock) fear conditioning.

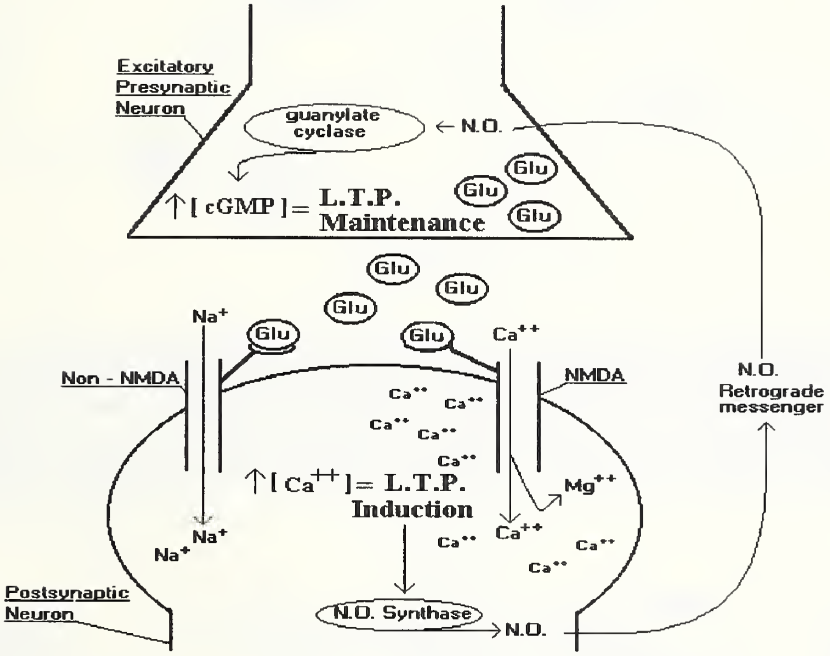


Fig. 1. Schematic diagram depicting the theoretical intracellular and intercellular signals and second messengers involved in LTP induction and maintenance. Glutamate released by an excitatory presynaptic neuron binds NMDA and non - NMDA receptors on a postsynaptic neuron. The non - NMDA receptor allows a small inward Na+ current. If the postsynaptic cell has been recently depolarized, the Mg++ block of the channel coupled to the NMDA receptor will be removed and binding of glutamate leads to a Ca++ influx which triggers LTP. The postsynaptic Ca++ spike triggers the synthesis and release of nitric oxide (NO) which then diffuses to the presynaptic neuron where it stimulates guanylyl cyclase resulting in an increase in the concentration of cGMP, which is required for LTP maintenance.

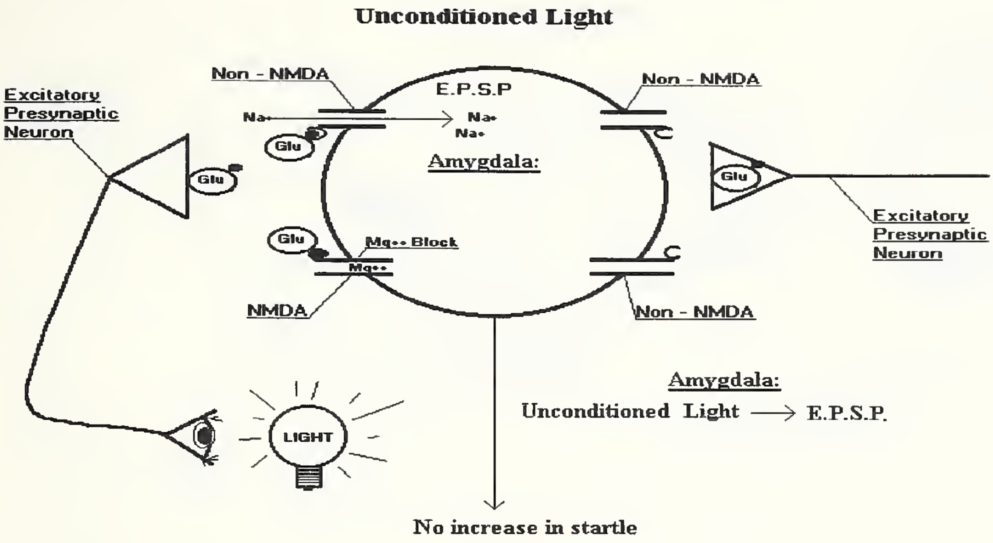


Fig. 2. Schematic diagram depicting the effect of an unconditioned light stimulus in the amygdala. An unconditioned light stimulates the release of glutamate (Glu) in the amygdala by excitatory presynaptic neurons receiving visual input. The glutamate binds to both NMDA and non - NMDA receptors. The non - NMDA receptors allow a small inward Na+ current which produces an equally small excitatory postsynaptic potential (EPSP). The NMDA receptor's ion channel is blocked by a Mg++ ion and nothing occurs.

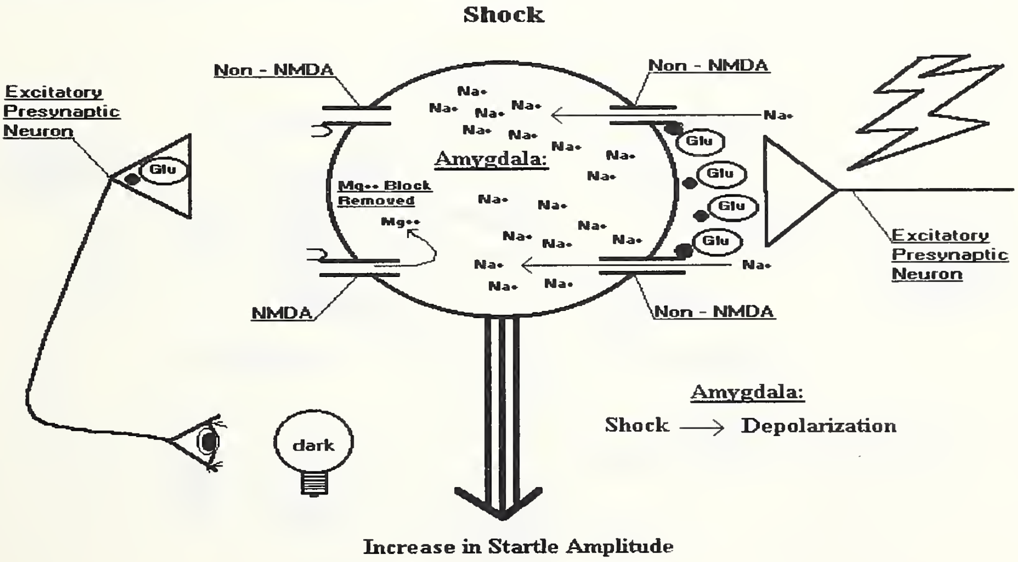


Fig. 3. Schematic diagram depicting the effect of a shock stimulus in the amygdala. A shock stimulates the release of glutamate (Glu) in the amygdala by excitatory presynaptic neurons. The glutamate binds to non - NMDA receptors and leads to a Na+ influx, depolarizing cells in the amygdala. The NMDA receptor's Mg++ block is removed

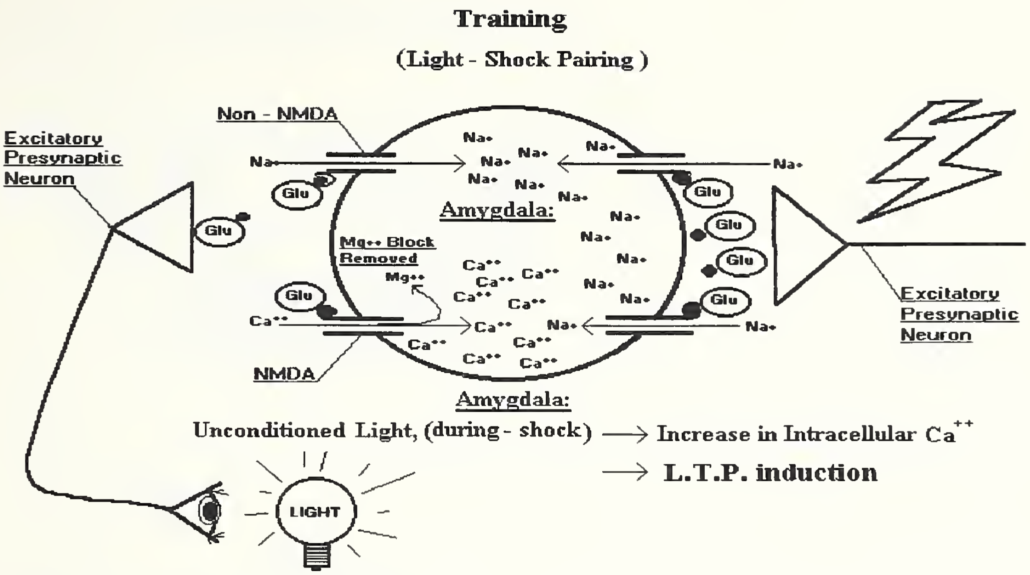


Fig. 4. Schematic diagram depicting the effect of light - shock pairing in the amygdala. A shock stimulates the release of glutamate (Glu) in the amygdala which binds to non - NMDA receptors and leads to a Na+ influx, depolarizing cells in the amygdala. The NMDA receptor's Mg++ block is removed. An unconditioned light stimulates the release of glutamate (Glu) in the amygdala which binds to both NMDA and non - NMDA receptors. The non - NMDA receptors allow a small inward Na+ current. The NMDA receptor allows a Ca++ influx which triggers LTP.

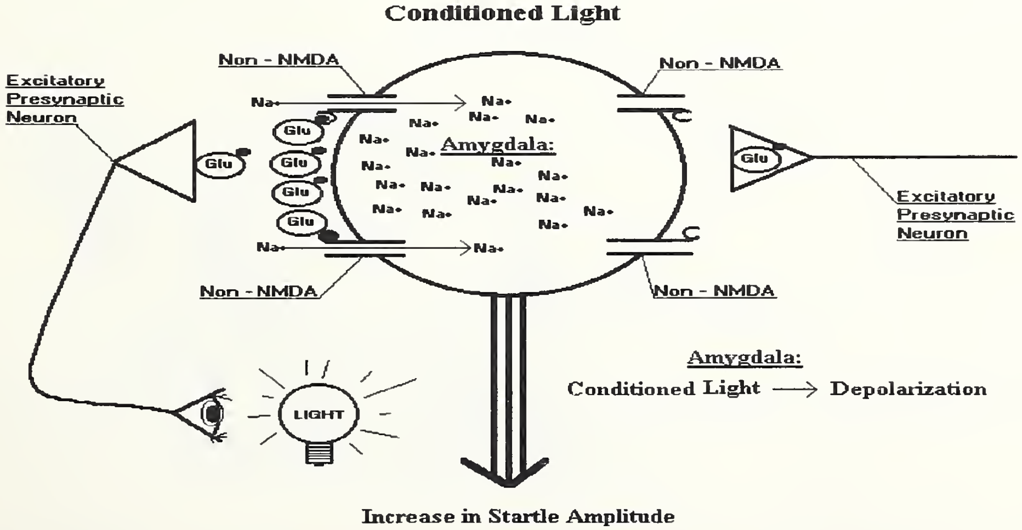
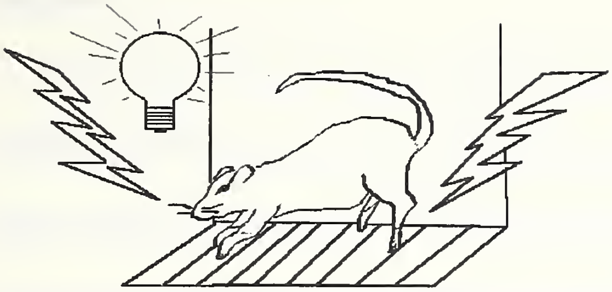
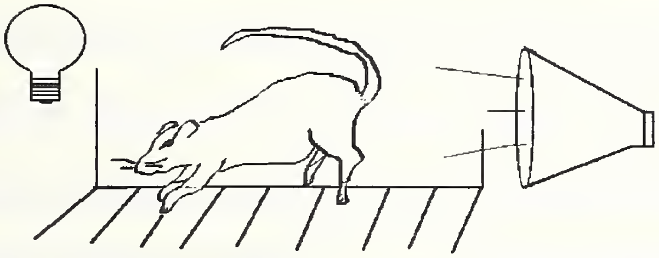


Fig. 5. Schematic diagram depicting the effect of a conditioned light stimulus in the amygdala. A conditioned light stimulates the release of glutamate (Glu) in the amygdala which binds to non - NMDA receptors and leads to a Na+ influx, depolarizing the cells in the amygdala. After training the visual input - amygdala synapse has increased efficacy such that visual stimulation alone activates the amygdala.

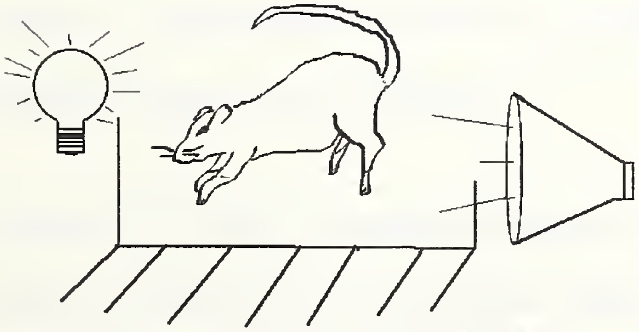
TRAINING: Light shock pairing



TESTING:



STARTLE ALONE (in the dark)



LIGHT - STARTLE (fear potentiated startle)

Fig. 6. Cartoon depicting the fear-potentiated startle paradigm. During training an unconditioned stimulus, shock, is repeatedly paired with a neutral stimulus, light. During testing startle is elicited by an auditory stimulus in both the presence and absence of the conditioned stimulus.

Materials and Methods

Apparatus

Fear conditioning and fear potentiated startle testing were conducted in five identical stabilimeter devices that have been described previously (Cassella & Davis, 1986). Briefly, each stabilimeter consisted of an 8 cm X 15 cm X 15 cm Plexiglas and wire mesh cage suspended between compression springs within a steel frame. The floor of each stabilimeter consisted of four, 6.0-mm diameter stainless steel bars spaced 18 mm apart which delivered shock. Cage movement resulted in displacement of an accelerometer with the resulting voltage being proportional to the velocity of displacement. The analog output of the accelerometer was amplified and digitized. Startle amplitude was defined as the peak accelerometer voltage that occurred during the first 200 ms after the onset of the startle stimulus. The stabilimeters were housed in a dimly lit, ventilated, sound attenuating chamber. A surveillance camera was positioned behind each stabilimeter to allow observation of the animals. Background noise (70 dB) was provided by a white noise generator. The light conditioned stimulus was produced by an 8 - W fluorescent bulb attached to the back of each stabilimeter.

Experiment #1. Effect of intraamygdala infusion of LY-83583, an inhibitor of cGMP production (Brandt et al 1991), during light - shock training, on the acquisition of fear conditioning as measured by fear potentiated startle.

Procedure

Twenty male, albino Sprague - Dawley rats weighing between 350 and 450g were anesthetized with Nembutal 50mg/cc, 0.125cc /100g body weight, and placed into a

stereotaxic instrument fitted with blunt ear bars. The skull was exposed and two holes were drilled above the amygdala at the following coordinates relative to bregma: anterior = -2.8; lateral = +/- 5.3. Cannulae (22 gauge outer cannulae, 26 gauge guide stylet) were lowered to -8.5 mm and glued to the skull with Loctite adhesive. Four 0-80 jewelers screws were placed in the skull, one anterior and one posterior to each cannula, and dental cement was then applied over the skull to stabilize and secure the cannulae. Each cannula was then covered with a screw on dust cap. One animal died at surgery. Twelve days after surgery 18 animals were matched, i.e. exposed to 30 startle alone stimuli consisting of a either a 100 dB, 105 dB, 110 dB, 50 ms burst of white noise presented in balanced irregular order with a 30 s inter-stimulus interval and divided into two groups having similar mean baseline startle amplitudes. One animal was excluded because of an aberrantly high baseline startle. One day later eight animals were infused with 2.5 nmol LY-83583 in 0.3 µl of PBS bilaterally, and nine animals with PBS alone as a control. The infusions occurred over 3 min. Injection cannulae remained in place for 1 min after injection. The 26 gauge stylets were then inserted into the cannulae, dust caps were replaced, and the animals were then placed into the startle apparatus. Training consisted of a 5-min acclimation period, followed by 30 light - shock pairings which consisted of a 3700 ms light paired with a 500 ms, 0.6mA shock presented 3200 ms after the light onset. The inter-trial interval alternated between 45 s, 60 s, and 75 s. Two days later the animals were tested for the acquisition of fear potentiated startle. Testing consisted of a 5-min acclimation period followed by 30 startle alone stimuli randomly mixed with 30 light - startle trials where the startle stimulus was delivered 3200 ms after light onset. Startle

stimuli consisted of a either a 100 dB, 105 dB, 110 dB, 50 ms burst of white noise presented in balanced irregular order with a 30 s inter-trial interval.

Experiment #2: Effect of intraamygdala infusion of NBQX on the expression of fear potentiated startle, in animals which previously received LY-83583 in experiment #1.

Procedure

Six days after their last training session and four days after their last test session, the 18 rats used in experiment #1 were briefly retrained for fear potentiated startle. Brief training consisted of 10 light -shock trials performed as previously described. The next day the animals were briefly re-tested for the acquisition of fear potentiated startle receiving 3 startle alone and 3 light startle trials as previously described. The following day the same eight animals that previously received LY-83583 were infused with 0.3 μg NBQX in PBS bilaterally. The nine control animals again received PBS. All animals were then tested for fear potentiated startle as previously described.

Experiment #3: Effect of various foot shock intensities on the acquisition of conditioned fear as measured by fear potentiated startle.

Procedure

Twenty male, albino Sprague - Dawley rats weighing between 250 and 350g were matched as previously described and divided into four groups having similar mean baseline startle amplitudes. One day after matching the animals were trained for fear potentiated startle as previously described. Different groups were trained at shock intensities of either

0.0 mA, 0.2 mA, 0.3 mA, or 0.4 mA. Two days after training, the animals were tested for fear potentiated startle as previously described. This experiment was then repeated with 20 new animals and the data combined.

Experiment #4: Effect of intraamygdala infusion of 8 Br-cGMP during weak (0.2 mA) shock training, on the acquisition of conditioned fear as measured by fear potentiated startle.

Procedure

Twenty two male, albino Sprague - Dawley rats weighing between 400 and 500g were implanted with bilateral amygdala cannulae as previously described. Four animals died immediately post-op and four animals lost their cannulae during the postoperative recovery period. Twenty days after surgery 14 animals were matched as previously described. Four days later seven animals were infused with 50 nM 8 Br-cGMP in ACSF bilaterally, and seven animals with ACSF alone as a control. The infusions and training were performed as previously described except that shock intensity was 0.2mA. Two days later the animals were tested for fear potentiated startle as previously described in experiment 1.

Experiment #5: Effect of intraamygdala infusion of 8 Br-cGMP during strong shock (0.6mA) training, on the acquisition of conditioned fear as measured by fear potentiated startle.

Procedure:

Two days after their last test session the seven animals that received 8 Br-cGMP in experiment #5 again were infused with 50 nM 8 Br-cGMP in ACSF bilaterally. Similarly, six of the seven animals who received ACSF alone in experiment #5 were again infused with ACSF as a control. One animal was excluded because of an aberrantly high baseline startle. Infusions and training occurred as previously described with a shock intensity of 0.6mA. Seventeen days after training the animals were tested for fear potentiated startle as previously described.

RESULTS

Experiment 1

Four animals did not receive shock during training because of a mechanical problem and were excluded from the data analysis. Fig. 7 shows the mean startle amplitude in the remaining animals for startle alone and light startle trials in animals receiving an intraamygdala infusion of either LY-83583 or phosphate buffered saline (PBS) during training. Bilateral intraamygdala infusion of 2.5 nmol LY-83583, 6 min. prior to 30 light - shock (0.6mA) pairings, had no effect on the acquisition of conditioned fear as measured by fear potentiated startle two days later. Animals that had received LY-83583 demonstrated a 32% (± 6%) increase in startle amplitude during light-startle trials. Similarly animals that had received PBS demonstrated a 31%(± 7%) increase in startle amplitude during light-startle trials. Analysis of variance of these data using trial type (Light - Startle vs. Startle alone) as a within-subjects factor and drug exposure (LY-83583 vs. PBS) as a between-subjects factor revealed a significant effect of trial type ($F_{1,12} = 33.820$, $P <.001$) indicating fear potentiated startle. However, there was no significant drug by trial type interaction ($F_{1,12} = 0.060$, $P <0.811$) indicating LY-83583 had no effect on the acquisition of fear potentiated startle. Subsequent individual t-tests performed on the two trial types found significant enhancement of startle by the light in the PBS group ($t_6 = 4.134$, $P <0.01$) as well as in the LY-83583 group ($t_6 = 4.100$, $P <0.01$).

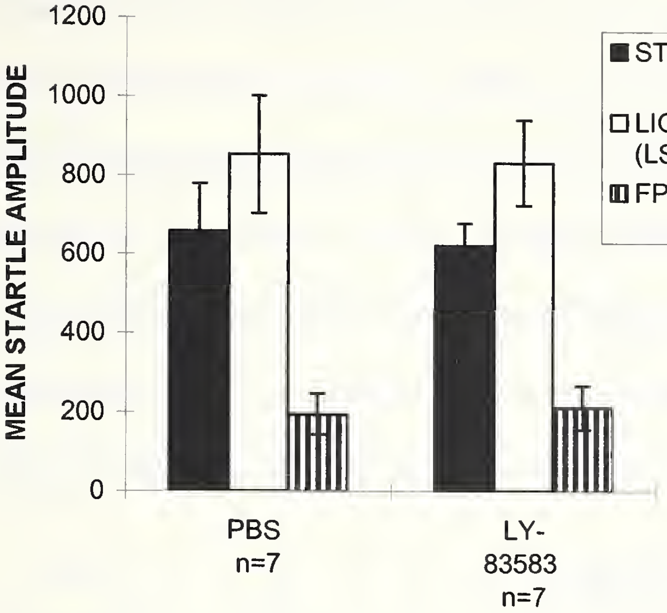
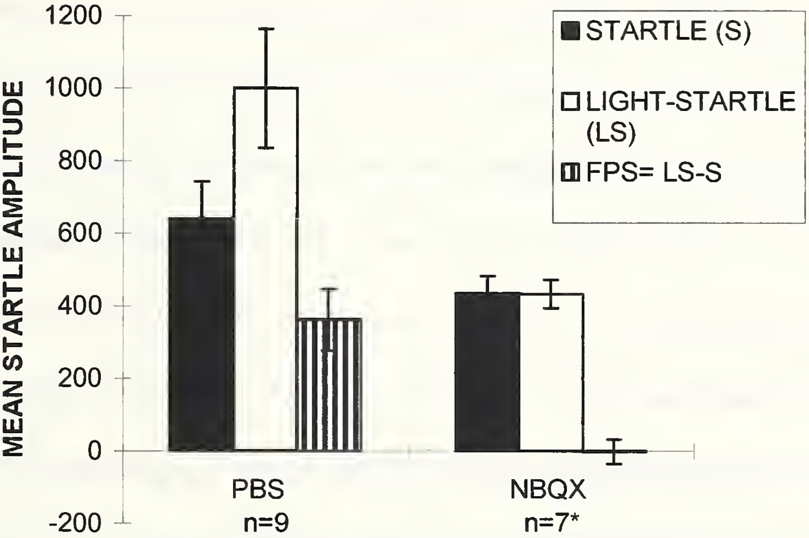


Fig. 7. Test for the acquisition of conditioned fear as measured by fear potentiated startle in animals receiving LY-83583 during (0.6mA) light-shock training. Black bars represent the mean amplitude of the acoustic startle reflex alone. White bars represent the mean amplitude of the acoustic startle reflex when elicited in the presence of a fear conditioned light stimulus. Stripped bars represent the mean difference between the startle alone and light-startle trials, defined as fear potentiated startle. Error bars represent ± S.E.M.

Experiment 2

Fig. 8 shows the mean startle amplitude for startle alone and light startle trials in the animals from experiment #1 receiving an intraamygdala infusion of either NBQX or PBS prior to re-testing for fear potentiated startle. Bilateral intraamygdala infusion of 0.3 μg of NBQX, 6 min prior to testing completely blocked the expression of fear potentiated startle in animals who previously received LY-83583 in experiment #1, demonstrating correct cannulae placement and effective drug delivery. Animals that had received NBQX demonstrated no increase, 2%(±8%), in startle amplitude during light-startle trials. Animals that had received PBS demonstrated a 60% (±14%), increase in startle amplitude during light-startle trials. Analysis of variance of these data using trial type (Light - Startle

vs. Startle alone) as a within-subjects factor and drug exposure (NBQX vs. PBS) as a between - subjects factor revealed a significant effect of trial type ($F_{1,14} = 14.220$, $P < .002$), indicating fear potentiated startle, as well as a significant drug by trial-type interaction ($F_{1,14} = 14.539$, $P < 0.002$), reflecting the differential effect of the light in the PBS vs. NBQX treated animals. Subsequent individual t-tests performed on the two trial types found significant enhancement of startle by the light in the PBS group ($t_8 = 4.529$, $P < 0.001$) but not in the NBQX group ($t_6 = 0.063$, $P < 0.475$).



*** Animals Receiving NBQX previously received LY-83583**

Fig. 8. Re-test for the expression of fear potentiated startle in animals from experiment #1 receiving either NBQX or PBS during re-testing. Black bars represent the mean amplitude of the acoustic startle reflex alone. White bars represent the mean amplitude of the acoustic startle reflex when elicited in the presence of a fear conditioned light stimulus. Stripped bars represent the mean difference between the startle alone and light-startle trials, defined as fear potentiated startle. Error bars represent ± S.E.M.

Experiment 3

Fig. 9 shows the mean startle amplitude for startle alone and light startle trials in animals receiving either a 0.0mA, 0.2mA, 0.3mA, or 0.4mA shock during training. Animals receiving light - shock training at 0.0mA (no shock) demonstrated an average 9% (±15%) increase in startle amplitude during light-startle trials over baseline startle

alone trials. Of the nine animals receiving 0.0mA (no shock), five demonstrated a fear potentiated startle of ≤10% (±15%) of baseline (Fig.10). Animals receiving light - shock training at 0.2mA intensity demonstrated an average 40% (±16%) increase in startle amplitude during light-startle trials. Five of the nine animals receiving 0.2mA shock, demonstrated a fear potentiated startle of ≤ 10%(±16%) (Fig.11). Animals receiving light - shock training at 0.3mA intensity demonstrated an average 46%(±10%) increase in startle amplitude during light-startle trials, and finally animals receiving light - shock training at 0.4mA intensity demonstrated an average 57%(±12%) increase in startle amplitude during light-startle trials. All 18 animals receiving either 0.3mA or 0.4mA shock during training demonstrated a fear potentiated startle of >10% (±10 %, ±12% respectively) (Fig. 12,13). Therefore 0.2mA, which produced ≤ 10% fear potentiated startle in more then 50% of animals, was adopted as an intensity which produced only weak fear conditioning. Analysis of variance of these data using trial type (Light - Startle vs. Startle alone) as a within-subjects factor and shock intensity (0.0mA, 0.2mA, 0.3mA, 0.4mA) as a between - subjects factor revealed a significant effect of trial type ($F_{1,32} = 47.768$, $P < .001$) indicating fear potentiated startle, as well as a significant shock intensity by trial-type interaction ($F_{1,32} = 3.888$, $P < 0.018$), reflecting the differential effect of the light in animals receiving different training shock intensities. Subsequent individual t-tests performed on the two trial types found significant enhancement of startle by the light in all groups receiving shock, 0.2mA ($t_7 = 2.560$, $P < 0.038$), 0.3mA ($t_8 = 6.082$, $P < 0.001$), 0.4mA($t_8 = 5.677$, $P < 0.001$).

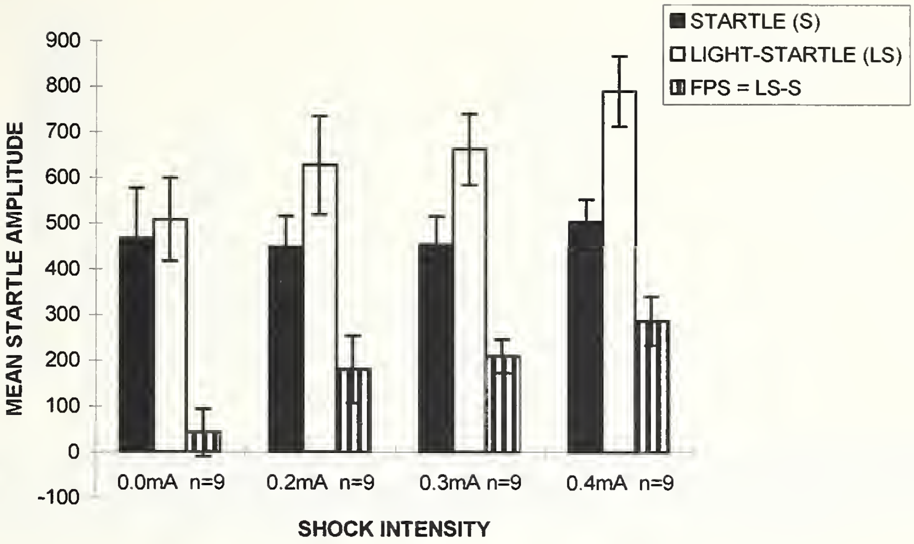


Fig. 9. Test for the expression of fear potentiated startle in animals receiving various shock intensities during fear conditioning. Black bars represent the mean amplitude of the acoustic startle reflex alone. White bars represent the mean amplitude of the acoustic startle reflex when elicited in the presence of a fear conditioned light stimulus. Stripped bars represent the mean difference between the startle alone and light-startle trials, defined as fear potentiated startle (fear potentiated startle). Error bars represent ± S.E.M.

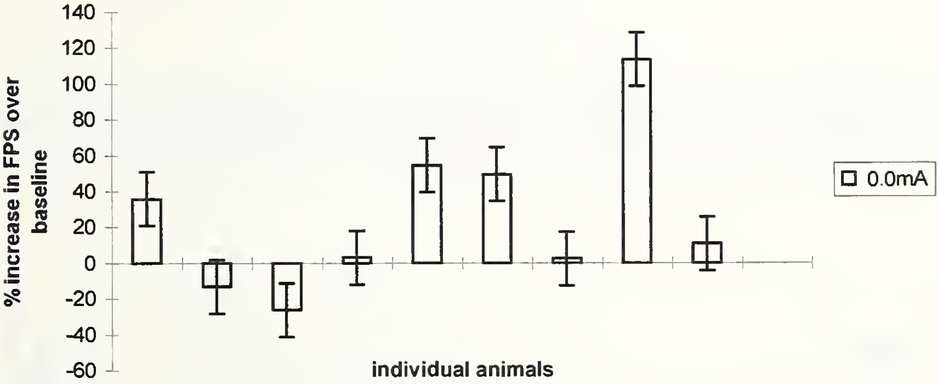


Fig. 10. % increase in fear potentiated startle over baseline startle in animals receiving 0.0mA, no shock, sham fear conditioning. White bars represent the mean % increase in fear potentiated startle over baseline. Data displayed by individual animal, n=9. Error bars represent ± S.E.M.

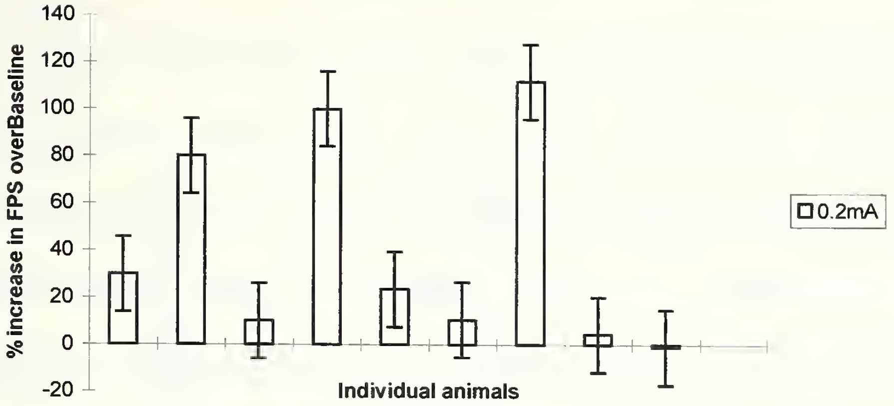


Fig. 11. % increase in fear potentiated startle over baseline startle in animals receiving 0.2mA footshock fear conditioning. White bars represent the mean % increase in fear potentiated startle over baseline. Data displayed by individual animal, n=9. Error bars represent ± S.E.M.

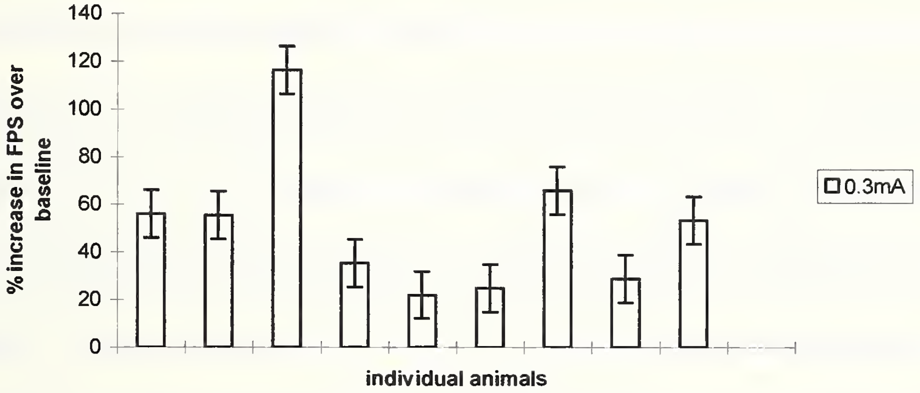


Fig. 12. % increase in fear potentiated startle over baseline startle in animals receiving 0.3mA footshock fear conditioning. White bars represent the mean % increase in fear potentiated startle over baseline. Data displayed by individual animal, n=9. Error bars represent ± S.E.M.

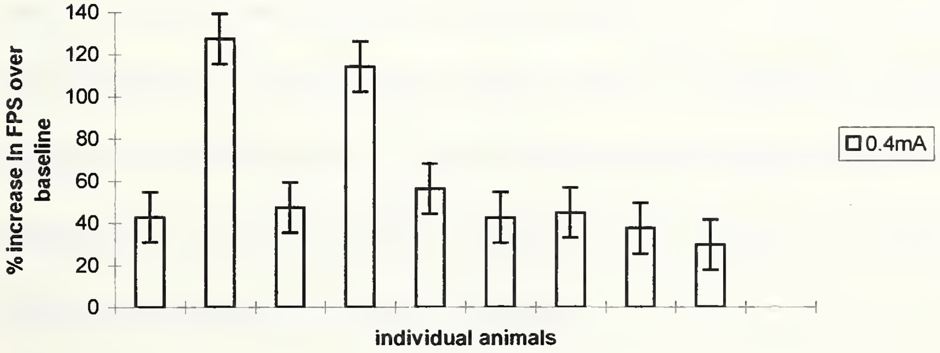


Fig. 13. % increase in fear potentiated startle over baseline startle in animals receiving 0.4mA footshock fear conditioning. White bars represent the mean % increase in fear potentiated startle over baseline. Data displayed by individual animal, n=9. Error bars represent ± S.E.M.

Experiment 4

Fig. 14 shows the mean startle amplitude for startle alone and light startle trials in animals receiving an intraamygdala infusion of either 8 Br-cGMP or artificial cerebral spinal fluid (ACSF) prior to weak fear conditioning. Bilateral intraamygdala infusion of 50nM 8 Br-cGMP, 6 min. prior to 30 light - (0.2mA) shock pairings, had no effect on the acquisition of weak fear conditioning as measured by fear potentiated startle two days later. Animals that had received 8 Br-cGMP demonstrated a 28% (±12%) increase in startle amplitude during light-startle trials. Similarly, animals that had received ACSF demonstrated a fear potentiated startle 12% (±8%) of baseline. Three of the six animals receiving 8 Br-cGMP, demonstrated a fear potentiated startle of $\leq$ 10%(±8%) of baseline (Fig. 16). Five of the six animals receiving ACSF, demonstrated a fear potentiated startle of $\leq$ 10%(±12%) of baseline (Fig. 15). Analysis of variance of these data using trial type (Light - Startle vs. Startle alone) as a within-subjects factor and drug exposure (8 Br-cGMP vs. ACSF) as a between-subjects factor failed to reveal a significant effect of trial type ($F_{1,12} = 0.584$, $P < .453$) failing to demonstrate fear potentiated startle. Similarly, there was no significant drug by trial type interaction ($F_{1,20} = 0.078$, $P < 0.782$) indicating 8 Br-cGMP had no effect on the acquisition of fear potentiated startle. Subsequent individual t-tests performed on the two trial types again failed to demonstrate significant enhancement of startle by the light in either the ACSF group (t5 = 1.3760, P <0.228) or the 8 Br-cGMP group ($t_5 = 2.421$, $P<0.068$).

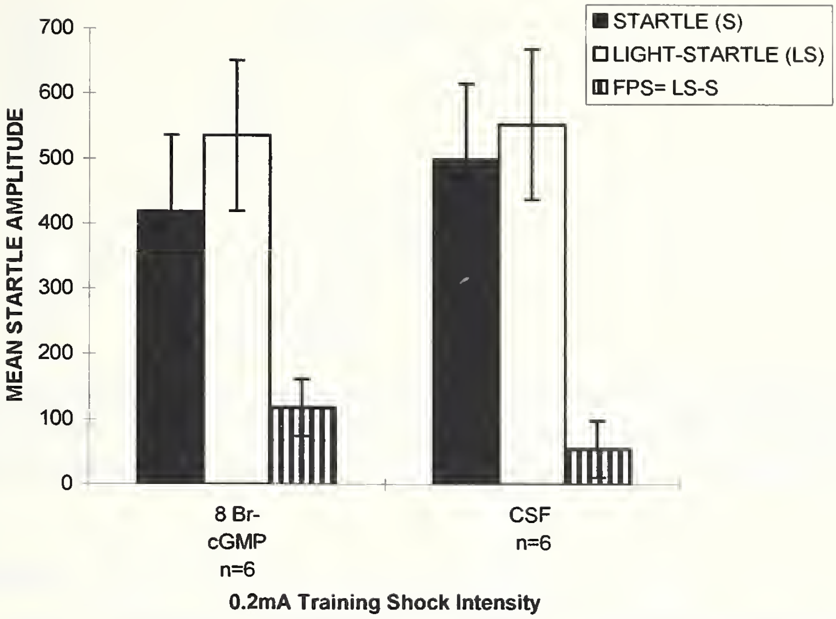


Fig. 14. Test for the expression of fear potentiated startle in animals receiving either 8 Br-cGMP or artificial cerebral spinal fluid (ACSF) during weak (0.2mA) fear conditioning. Black bars represent the mean amplitude of the acoustic startle reflex alone. White bars represent the mean amplitude of the acoustic startle reflex when elicited in the presence of a fear conditioned light stimulus. Stripped bars represent the mean difference between the startle alone and light-startle trials, defined as fear potentiated startle. Error bars represent ± S.E.M.

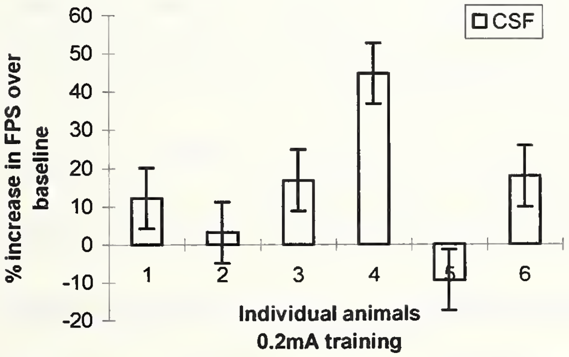


Fig. 15. % increase in fear potentiated startle over baseline startle in animals receiving ACSF during weak (0.2mA) fear conditioning. White bars represent the mean % increase in fear potentiated startle over baseline. Data displayed by individual animal, n=6. Error bars represent ± S.E.M.

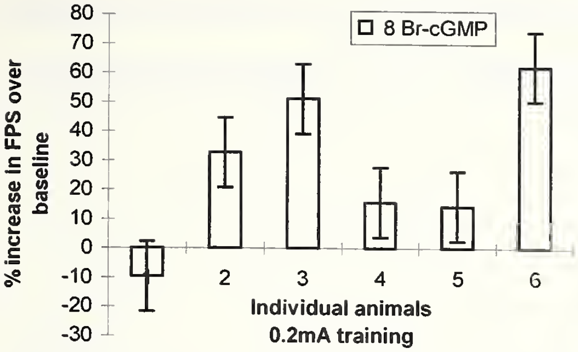


Fig. 16. % increase in fear potentiated startle over baseline startle in animals receiving 8 Br-cGMP during weak (0.2mA) fear conditioning. White bars represent the mean % increase in fear potentiated startle over baseline. Data displayed by individual animal, n=6. Error bars represent ± S.E.M.

Experiment 5

Fig. 17 shows the mean startle amplitude for the first 60 startle alone and light startle trials in animals receiving an intraamygdala infusion of either 8 Br-cGMP or artificial cerebral spinal fluid (ACSF) prior to strong fear conditioning. Bilateral intraamygdala infusion of 50nM 8 Br-cGMP, 6 min. prior to 60 light - shock (0.6mA) pairings, had no effect on the acquisition of strong fear conditioning as measured by fear potentiated startle 17 days later. Animals that had received 8 Br-cGMP demonstrated a 58% (±10%) increase in startle amplitude during light-startle trials. Similarly, animals that had received ACSF demonstrated a fear potentiated startle 69% (±16%) of baseline. Analysis of variance of these data using trial type (Light - Startle vs. Startle alone) as a within-subjects factor and drug exposure (LY-83583 vs. PBS) as a between-subjects factor revealed a significant effect of trial type ($F_{1,20} = 5.977$, $P < .024$) indicating fear potentiated startle. However, there was no significant drug by trial type interaction ($F_{1,20} = 0.001$, $P < 0.977$) indicating 8 Br-cGMP had no effect on the acquisition of fear potentiated startle. Subsequent individual t-tests performed on the two trial types found

significant enhancement of startle by the light in the ACSF group ($t_5 = 3.550$, $P < 0.02$) as well as in the 8 Br-cGMP group ($t_6 = 9.700$, $P < 0.001$).

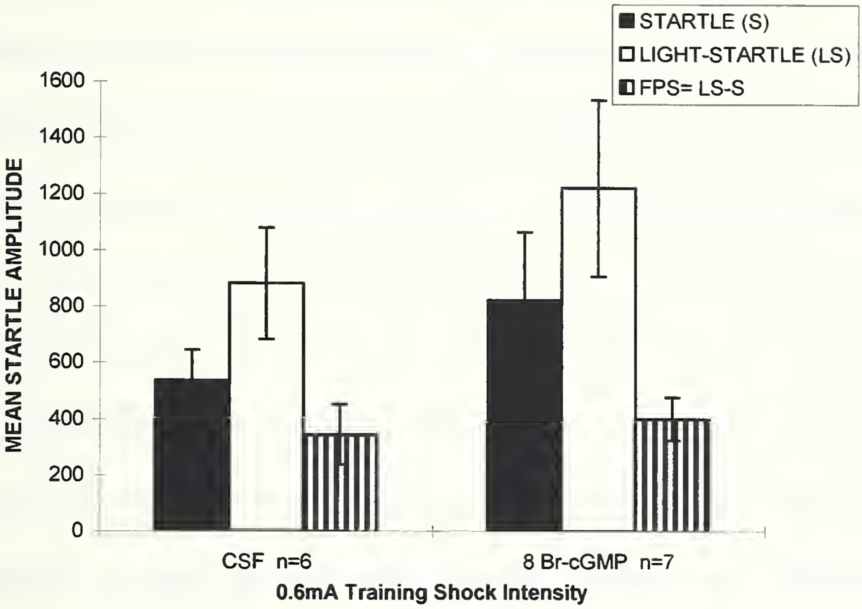


Fig. 17. Test for the expression of fear potentiated startle in animals receiving either 8 Br-cGMP or artificial cerebral spinal fluid (ACSF) during strong (0.6mA) fear conditioning. Black bars represent the mean amplitude of the acoustic startle reflex alone. White bars represent the mean amplitude of the acoustic startle reflex when elicited in the presence of a fear conditioned light stimulus. Stripped bars represent the mean difference between the startle alone and light-startle trials, defined as fear potentiated startle. The 7 animals receiving 8 Br-cGMP, and the six controls, are the same animals that received 8 Br-cGMP and ACSF respectively in experiment #4. Error bars represent ± S.E.M.

Discussion

Recent studies have implicated cGMP as a crucial second messenger involved in LTP. Because LTP may ultimately involve an increase in presynaptic transmitter release, various retrograde messengers have been proposed to explain how events in the post-synaptic cell can affect the presynaptic cell. In addition to NO (see introduction), other putative retrograde messengers such as carbon monoxide and arachidonic acid (Williams et al 1989) are known to be coupled to cGMP. Hence treatments that alter cGMP would be expected to alter LTP, regardless of the retrograde messenger, which may vary from brain area to brain area. Addition of 8 Br-cGMP paired with weak presynaptic stimulation has been shown to induce LTP in at least two distinct in vitro models (Bennett et al 1993; Hawkins et al 1994). Similarly, inhibitors of cGMP such as LY-83583 have been shown to inhibit LTP (Zhuo et al 1994). The present experiments were conducted in an attempt to demonstrate the role of cGMP in a behavioral model of learning, namely fear conditioning. Fear conditioning was measured via the fear potentiated startle paradigm, where the strength of learning is related to the amplitude of the acoustic startle reflex when elicited in the presence of a fear conditioned light stimulus.

Bilateral intraamygdala infusion of 2.5 nmol LY-83583, prior to light - shock (0.6mA) pairing, had no effect on fear conditioning, as measured by the fear potentiated startle paradigm. The subsequent infusion of NBQX and re-test for the expression of fear potentiated startle demonstrated correct cannulae placement and effective drug delivery in animals previously infused with LY-83583.

Next we attempted to enhance fear conditioning. In vitro studies of both the rat hippocampus and the chick ciliary ganglion have demonstrated an increase in LTP after the administration of 8 Br-cGMP, when paired with weak presynaptic stimulation (Lin et al 1994; Hawkins et al 1994).We generated an intensity response curve to determine a shock intensity which produced only weak fear conditioning, reasoning that a weak shock would be equivalent to the weak presynaptic stimulation used in the in vitro models.

As anticipated, the magnitude of fear conditioning increased with increasing shock intensity. The mean fear potentiated startle amplitude for the 0.2mA, 0.3mA, and 0.4mA groups all had overlap with respect to SEM, yet examination of the data by individual animal revealed differences among the groups. 100% of animals receiving either 0.3mA or 0.4mA shock demonstrated a fear potentiated startle >10% of baseline, where as only 33% of animals receiving 0.2mA shock, demonstrated a fear potentiated startle of > 10% of baseline. Therefore 0.2mA was adopted as an intensity which produced only weak fear conditioning.

Bilateral intraamygdala infusion of 0.3 µl 50nM 8 Br-cGMP, prior to light - (0.2mA) shock pairings, had no effect on the acquisition of weak fear conditioning. When examined by individual animal the group receiving 8 Br-cGMP, was similar to both the ACSF controls and the 0.2mA shock alone group from experiment #3, suggesting 8 Br-cGMP had no effect. We chose 0.2mA because we felt it was a threshold intensity and thus would be a sensitive place to look for learning enhancement. Our failure to demonstrate significant fear potentiated startle in either the ACSF or the 8 Br-cGMP group may suggest that the intraamygdala infusion produced some degree of pressure

damage effectively raising this threshold. In light of this, 0.3mA may have been a more appropriate intensity.

Finally we attempted to enhance strong fear conditioning. Bilateral intraamygdala infusion of 0.3 μl 50nM 8 Br-cGMP, prior to light - shock (0.6mA) pairings, had no effect on the acquisition of strong fear conditioning as measured by mean fear potentiated startle amplitude.

Overall, we were not able to manipulate the acquisition of fear potentiated startle by intraamygdala infusion of agents shown to both impair and enhance in vitro LTP. One possibility is that LY-83583 was not effective in blocking cGMP in the amygdala. This compound has been shown to work in some tissues and not in others, and has had significantly better efficacy in vitro then in vivo (Bandt et al 1991). Other options were to attempt to inhibit the cascade upstream with NO synthase inhibitors or NO scavengers, or downstream with cGMP dependent protein kinase inhibitors. Recently, NO synthase inhibitors which successfully blocked LTP in vitro failed to do so in vivo (Bannerman et al 1994). Furthermore, prior work in this laboratory found NO synthase inhibitors to be toxic when infused directly into the amygdala. NO scavengers such as hemoglobin have a very high molecular weight and are difficult to work with in our model. A significant difficulty when moving from in vitro to in vivo work is determination of drug dose. To adequately rule out the in vivo efficacy of these agents one would have to repeat these experiments at other drug concentrations. In addition, measuring intraamygdala levels of cGMP was beyond the scope of this research. To definitively implicate the role cGMP in fear conditioning, one must be able to correlate intraamygdala cGMP levels with behavioral data.

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